Interaction of the Organochlorine Pesticide Dieldrin with Phospholipid Bilayers

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Pesticide, Dieldrin, Phospholipid Bilayer, Erythrocyte Membrane

Dieldrin is an organochlorine insecticide highly toxic for human beings. Although its exact mechanism of action is not well known, there is evidence that it acts at the cell membrane level. In fact, the lipophilicity of the pesticide as well as that of the phospholipid bilayer present in biological membranes makes the latter a most likely target for the interaction of dieldrin with living organisms. In order to evaluate its perturbing effect upon cell membranes the pesticide was made to interact with human erythrocytes and molecular models. These studies were performed by scanning electron microscopy on erythrocytes, fluorescence spectroscopy on dimyristoylphosphatidylcholine (DMPC) large unilamellar vesicles and X-ray diffraction on multilayers of dimirystoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE). It was observed that dieldrin particularly interacted with DMPC liposomes and multilayers perturbing its molecular arrangements. However, no effect was noticed on erythrocytes, which might be due to its high cholesterol content.

Introduction

The widespread use of pesticides in an attempt to increase crop production has generated a series of toxicological and environmental problems, particularly in developing countries (Newberne, 1989) Thus, their increased use in the last four decades has been accompanied by numerous cases of acute poisoning. Dieldrin, whose structural formula is shown in Fig. 1, is an organochlorine insecticide widely used for the control of many soil pests and

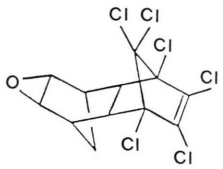

Fig. 1. Structural formula of dieldrin.

in the treatment of seed. Since the early 1970 it has been severely restricted or banned. Nevertheless, its use for the protection of wood against insects and termites continues in several countries. Dieldrin is highly toxic for human beings; its level in blood below which adverse effects do not occur is 105 μg/liter (Environmental Health Criteria 91, 1989). Although its exact mechanism of action is not well known, it has been suggested that it may act on the chloride channel of the γ-aminobutyric acid $(GABA)_A$ system (Nagata *et al.,* 1994). However, there is considerable evidence that its toxicity is related to alterations of membrane structure (Fonovich and Pechen, 1991). In fact, the perturbation of membrane lipids may be responsible, at least in part, for its toxic effects (Goel *et al.,* 1988). In general, the molecular mechanisms of pesticide action are poorly understood. However, the lipophilicity of most of them make lipid-rich membranes a plausible target of their interaction with living organisms (Blasiak, 1995). Some effects directly related to toxicity could be due to changes in membrane fluidity as a primary pesticide effect (Videira *et al.,* 1996). One of such effects is the DDT-induced change in the permeability of human erythrocyte lipid bilayer for electrolytes (Ahmad and Chefurka, 1982). This is consisting with the hypothesis that the alterations in the stiffness of lipid bilayers are likely to constitute a general mechanism for modulation of membrane protein functions (Lundbaeck *et al.,* 1996). Thus, it has been reported that dieldrin affects the activity of phospholipase C, effect explained by the insecticide perturbation of the lipid environment of the en-

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zyme (Fonovich and Pechen, 1995). However, in spite of the implications that at least part of the toxic effects of the pesticides could be due to perturbations of the lipid phase of membranes (Bach and Sela, 1984), a clear relationship between changes in fluidity evoked by pesticides and their toxicity has to be established.

This paper describes the results of our studies on the interaction of dieldrin with human erythrocyte membranes and models constituted by phospholipid multilayers and large unilamellar vesicles. These systems have previously been used in our laboratories to determine the interaction and perturbing effects on membranes by several therapeutic drugs (Suwalsky *et al.,* 1991, 1994) and the pesticides DDT (Suwalsky *et al.,* 1985), pentachlorophenol (Suwalsky *et al.,* 1990, 1992) and 2,4-D (Suwalsky *et al.,* 1996). The multilayers consisted in the phospholipids dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), which represent types of phospholipids respectively located in the outer and inner monolayers of the human erythrocyte membrane (Devaux and Zachowsky, 1994). Given the lipophilic nature of dieldrin and the amphiphilic character of both phospholipids, their interactions were assayed in a hydrophobic medium as well as in water under a wide range of concentrations. The capacity of dieldrin to perturb the multilayer structure of DMPC and DMPE was determined by X-ray diffraction methods.

Fluorescent steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the general polarization of 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) were determined in DMPC large unilamellar vesicles. DPH is one of the most used probes for the hydrophobic regions of phospholipid bilayers. Its fluorescence steady-state anisotropy provides a measure of the rotational diffusion of the fluorophore restricted within a certain region due to the phospholipid hydrocarbon chain order. On the other hand, Laurdan has a high sensitivity of excitation and emission spectra to the physical state of membranes (Parasassi and Gratton, 1995). With the fluorophore moiety located in a shallow position of the bilayer normal in the phospholipid polar head group environment, Laurdan provides information of dynamic properties in this zone of the bilayer (Chong, 1988, 1990). Laurdan spectral shift quantification is done using the general polarization (GP) concept (Parasassi and Gratton, 1995).

Finally, dieldrin was incubated with human erythrocytes which were later observed by scanning electron microscopy to detect possible shape changes induced by the insecticide.

Materials and methods

X-ray diffraction analysis of phospholipid multilayers

Synthetic DMPC (lot 80H8371, A grade, MW 677.9) and DMPE (lot 13H83681, A grade, MW 635.9) from Sigma and dieldrin (99%, MW 380.9) from Aldrich were used without further purification. About 3 mg of each phospholipid were mixed with the corresponding weight of dieldrin in order to attain DMPC:Dieldrin and DMPE:Dieldrin powder mixtures in the molar ratios of 10:1, 5:1, 2:1 and 1:1. Each mixture was dissolved in chloroform:methanol 3:1 v/v and left to dry. The recrystallized samples were introduced into special glass capillaries 0.7 mm diameter. They were diffracted in Debye-Scherrer cameras of 114.6 mm diameter and flat-plate cameras with 0.25 mm diameter glass collimators provided with rotating devices. The same procedure was also followed with samples of each phospholipid and dieldrin. The aqueous specimens were prepared in 1.5 mm diameter glass capillaries mixing each phospholipid and dieldrin in the same proportions as described above. Each capillary was then filled with about 200 μL of distilled water. These specimens were X-ray diffracted 2 days after preparation in flat-plate cameras. Specimen-to-film distances were 8 or 14 cm, standardized by sprinkling calcite powder on the capillaries surface. Ni-filtered CuKα radiation from a Philips PW1140 X-ray generator was used. The relative reflection intensities were obtained from films by peak-integration in a Joyce-Loebl MKIIICS microdensitometer interfaced to a PC. No correction factors were applied. The experiments in water were performed at 17 ± 2°C, which is below the main transition temperatures of both DMPC and DMPE.

Fluorescence measurements on large unilamellar vesicles (LUV)

DMPC LUV suspended in water were prepared by extrusion of frozen and thawed multilamellar

liposome suspension through two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Star Corp.) employing nitrogen pressure at 10 °C over the lipid transition temperature, to a final concentration of 0.3 mM. DPH and Laurdan were incorporated into LUV by addition of small aliquots of concentrated solutions of the probe in ethanol to LUV suspension in water and gently shaken by ca. 30 min. The final probe concentration was 0.5 μM. Fluorescence spectra and anisotropy measurements were performed in a Fluorolog spectrofluorometer from Spex and in a phase shift and modulation Greg-200 spectrofluorometer from ISS respectively, both interfaced to PC using ISS software. Measurements of LUV suspensions were made at 18 °C employing 10 mm path-length square quartz cuvettes. Sample temperature was controlled by an external Cole Parmer bath circulator and measured prior and after each measurement using an Omega digital thermometer. Anisotropy measurements were done in the "L" configuration using Glan Thompson prism polarizers in both exciting and emitting beams. The emission was measured with the aid of a WG-420 Schott high pass filter which showed negligible fluorescence. GP was evaluated by GP=(Ib-Ir)/(Ib+Ir), where Ib and Ir are the intensities at the blue and red edges of the emission spectrum, respectively. This parameter is used to quantitatively express Laurdan fluorescence spectrum maximum shifts due to the dipolar relaxation processes at the probe environment. A high GP value corresponds to a non-relaxed blue shifted spectrum while a low one corresponds to a relaxed red shifted spectrum. These intensities were measured at 440 and 500 nm, corresponding to the emission maxima of Laurdan in gel and liquid-crystalline phases respectively (Parasassi and Gratton, 1995). For both probes the excitation wavelength was set at 360 nm. Blanck suspensions without probe were used to correct background light scattering. Dieldrin was incorporated as small aliquots from a concentrated aqueous suspension.

Scanning electron microscope (SEM) studies on human erythrocytes

The interaction of dieldrin with human erythrocytes was achieved by incubating blood samples from clinically healthy male adult donors not being treated with any pharmacological agent by puncture of the ear lobule disinfected with 70% ethanol. Two drops were received in a plastic tube containing 10 ml of saline solution (0.9% NaCl) at 5 °C. This blood solution was used to prepare the following samples:

a) control, by mixing 1 ml with 9 ml of saline solution, and b) dieldrin suspensions equivalent to 0.1 mM up to 100 mM by mixing 1 ml of blood solution plus 9 ml of dieldrin saline suspensions of adequate concentrations. These samples were incubated at 37 °C for 1 h in an oven. They were then fixed with glutaraldehyde by adding one drop of each sample to a tube containing 1 ml of 2.5% glutaraldehyde in saline solution, reaching a final fixation concentration of about 2.4%. After resting overnight at 5 °C, the fixed samples were placed directly on Al stubs, air dried in an oven at 37 °C for half to one hour and gold coated for 3 min at 10–1 Torr in a S150 Edwards sputter device. The observations and photographic records were performed in an Etec Autoscan SEM.

Results

X-ray studies on phospholipid multilayers

The molecular interaction of dieldrin with multilayers of the phospholipids DMPC and DMPE were studied in hydrophobic and aqueous media. Table I shows the interplanar spacings and relative intensities of the reflections produced by DMPC, dieldrin and their 5:1, 2:1 and 1:1 molar mixtures after interacting and being recrystallized from chloroform:methanol 3:1 v/v solutions. Their respective diffractograms are compared in Fig. 2 (a). The analysis of these results indicated that the X-ray pattern of DMPC was affected by increasing concentrations of dieldrin. In fact, at the DMPC:Dieldrin molar ratio of 5:1 the reflection intensities of the lipid became weaker, disappearing many of them at the 1:1 ratio. On the other hand, two new reflections of 7.00 Å and 6.19 Å showed up in the 2:1 and 1:1 specimens. They corresponded to the two strongest reflections of dieldrin. On the other hand, the bilayer width of DMPC remained practically constant at about 55 Å. These results indicated that part of dieldrin interacted with DMPC, deeply penetrating into the phospholipid bilayer and perturbing its structure.

Table I. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylethanolamine (DMPE), dieldrin and of their 5:1, 2:1 and 1:1 molar mixtures [a-c].

DMPC : Dieldrin									
DMPC		5 : 1		2 : 1		1 : 1		Dieldrin	
do[Å]	Io rel	do[Å]	Io rel	do[Å]	Io rel	do[Å]	Io rel	do[Å]	Io rel
54.3*	344*	54.7*	134*	54.5*	126*	54.5*	128*	--	--
27.2*	6*	27.2*	5*	27.2*	5*	27.2*	3*	--	--
18.5	2	--	--	--	--	--	--	--	--
13.7	14	13.7	7	13.7	7	13.5	5	--	--
9.30	4	--	--	--	--	--	--	--	--
8.30	2	--	--	--	--	--	--	--	--
--	--	--	--	6.98	2	6.98	5	7.00	12
6.29	10	6.28	7	6.27	10	--	--	--	--
--	--	--	--	--	--	6.22	15	6.19	25
4.66	6	--	--	--	--	--	--	--	--
4.32	36	4.29	12	4.29	7	4.30	2	--	--
4.13	100	4.13	50	4.13	48	4.13	30	--	--
3.88	23	3.86	3	3.85	3	3.85	3	3.83	10
DMPE : Dieldrin									
DMPE		5 : 1		2 : 1		1 : 1		Dieldrin	
do[Å]	Io rel	do[Å]	Io rel	do[Å]	Io rel	do[Å]	Io rel	do[Å]	Io rel
51.4	768	51.2	640	51.2	633	51.4	633	--	--
25.5	3	25.5	3	--	--	--	--	--	--
17.0	3	17.1	3	17.0	2	17.0	2	--	--
12.8	5	12.8	4	12.8	3	12.8	3	--	--
--	--	7.00	3	7.00	4	7.00	7	7.00	12
--	--	6.18	5	6.18	7	6.19	14	6.19	25
5.95	12	5.95	10	5.96	3	5.96	2	--	--
5.69	7	5.69	6	--	--	--	--	--	--
4.78	16	4.78	14	4.78	12	4.78	10	--	--
4.66	14	4.65	12	4.65	8	4.66	6	--	--
4.04	99	4.04	100	4.04	51	4.04	47	--	--
3.80	59	3.81	54	3.82	27	3.83	31	3.83	10

[a] All the specimens were recrystallized from $CHCl_3$:CH_3OH 3:1 (v/v).
[b] The interplanar spacings and intensities of the reflections were measured in X-ray diagrams from Debye-Scherrer and flat-plate cameras with 8 and 14* cm specimen-to-film-distances.
[c] Only the main observed reflections are included.

Table II and Fig. 2 (b) show the results obtained after DMPC, dieldrin and their molar mixtures in the same ratios as above were immersed in distilled water. It was observed that water produced an expansion of DMPC bilayer width from about 55 Å when dry to nearly 63 Å. The observed reflections were reduced to only the first three orders of the bilayer width and a relatively intense one of about 4.2 Å. The latter arised from the stiff and fully extended hydrocarbon chains organized with rotational disorder in an hexagonal lattice (Janiak *et al.*, 1976). The increasing proportion of dieldrin in the mixtures produced a gradual diminishing of the phospholipid reflection intensities. This meant a perturbation of the DMPC bilayer structure despite the fact that dieldrin is very insoluble in water. Moreover, no reflections from the insecticide were observed even at its 1:1 molar ratio.

Table I and Fig. 3 (a), respectively, show the interplanar spacings and X-ray patterns obtained after DMPE was made to interact with dieldrin in the same way as described for DMPC in an hydrophobic medium. The perturbing effect of the

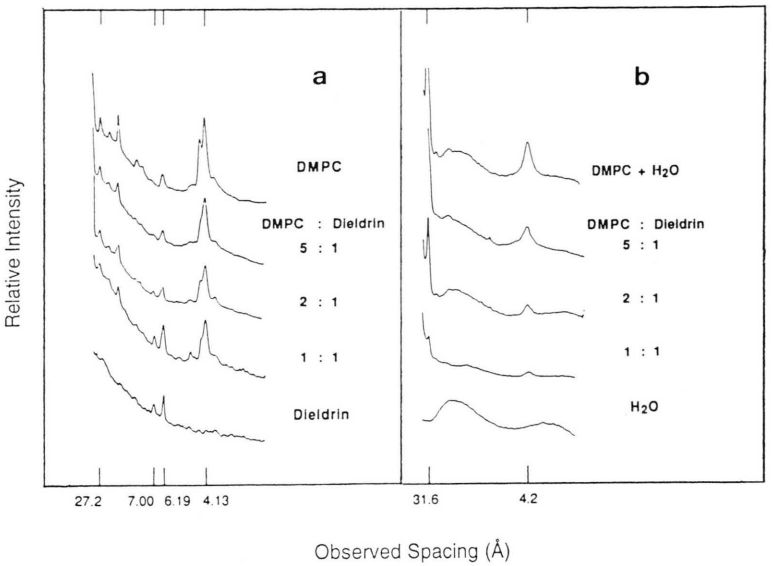

Fig. 2. Microdensitograms from X-ray diffraction diagrams of dimyristoylphosphatidylcholine(DMPC), dieldrin and of their 5:1, 2:1, 1:1 molar mixtures. Flat-plate cameras, specimen-to-film distance 8 cm. (a) Recrystallized from $CHCl_3$:CH_3OH 3:1 (v/v); (b) immersed in water.

Table II. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylethanolamine (DMPE), dieldrin and of their 5:1, 2:1 and 1:1 molar mixtures in the presence of water[a-b].

DMPE : Dieldrin									
DMPE		5 : 1		2 : 1		1 : 1		Dieldrin	
do[Å]	Io rel	do[Å]	Io rel	do[Å]	Io rel	do[Å]	Io rel	do[Å]	Io rel
63.1*	205*	63.1*	167*	63.3*	165*	--	--	--	--
31.6*	89*	31.6*	87*	31.6*	85*	--	--	--	--
21.0	5	21.1	3	--	--	--	--	--	--
--	--	--	--	--	--	--	--	7.00	19
--	--	--	--	--	--	--	--	6.19	31
4.2	100	4.2	59	4.2	35	4.2	4	--	--
--	--	--	--	--	--	--	--	3.83	10

DMPE		DMPE:Dieldrin 1 : 1		Dieldrin[c]	
do[Å]	Io rel	do[Å]	Io rel	do[Å]	Io rel
51.4*	1051*	51.3	473*	--	--
25.5*	2*	--	--	--	--
17.0	5	17.1	4	--	--
12.7	10	12.7	7	--	--
--	--	--	--	7.00	12
--	--	6.22	2	6.19	25
5.93	6	5.95	10	--	--
4.78	7	4.81	18	--	--
4.04	100	4.04	74	--	--
3.79	37	3.81	26	3.83	10

[a] The interplanar spacings and intensities of the reflections were measured in X-ray diagrams from flat-plate cameras with 8 and 14* cm specimen-to-film distances.
[b] Only the main observed reflections are included.
[c] Data from dry specimens.

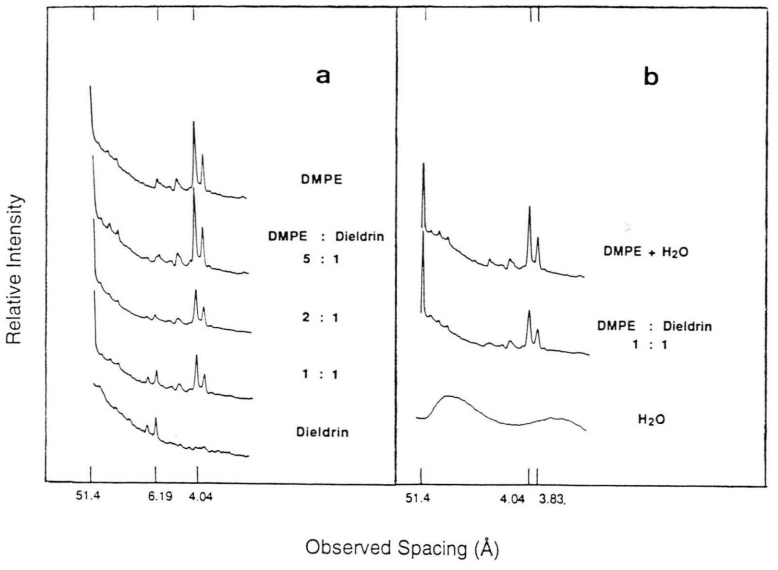

Fig. 3. Microdensitograms from X-ray diffraction diagrams of dimyristoylphosphatidylethanolamine (DMPE), dieldrin and of their 5:1, 2:1, 1:1 molar mixtures. Flat-plate cameras, specimen-to-film distance 8 cm. (a) Recrystallized from $CHCl_3$:CH_3OH 3:1 (v/v); (b) immersed in water.

pesticide upon the structure of DMPE multilayers was milder than that observed in DMPC. In fact, only at their 2:1 and 1:1 molar ratios it was observed a change in the lipid pattern which consisted in a diminishing of its reflection intensities. On the other hand, the two strongest reflections of dieldrin were also present in these mixture. Finally, the results of the interaction of DMPE and dieldrin in the presence of water are presented in Table II and Fig. 3(b). As it can be observed, at the highest molar ratio assayed the insecticide only produced a mild diminishing of DMPE reflection intensities. However, no reflections from dieldrin were observed.

Fluorescent measurements on large unilamellar vesicles (LUV)

The effect of dieldrin upon DMPC LUV was studied at the hydrocarbon chain and the hydrophilic/hydrophobic interface levels of the bilayer by respectively evaluating DPH steady state fluorescence anisotropy (r) and Laurdan general polarization (GP). As shown in Table III, the presence of increasing concentration of dieldrin produced a monotonous decrease in the fluorescence parameters of both probes. The DPH steady-state anisotropy is related primarily to the restriction of the rotational motion due to the hydrocarbon chain packing order. Therefore, the observed decrease in this parameter can be rationalized as a high structural disruption of the bilayer hydrophobic region produced by the incorporation of dieldrin. On the other hand, the effect of Laurdan GP indicates that the dynamics of the dipolar relaxation and/or the water penetration at the polar head group level was highly increased by the dieldrin incorporation. There is, therefore, an agreement between these results and those obtained by X-ray diffraction on DMPC multilayers in an aqueous medium.

Table III. Effect of dieldrin on the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the general polarization (GP) of Laurdan embedded in large unilamellar dimyristoylphosphatidylcholine (DMPC) vesicles. Probe: lipid ratio 1:600.

Dieldrin conc. [mM]	r DPH	GP Laurdan
0.00	0.272	0.513
0.01	0.244	0.405
0.10	0.108	0.093
1.00	0.016	--

Scanning electron microscopy (SEM) studies on human erythrocytes

The electron microscopic examination of blood samples incubated with dieldrin at several concen-

trations failed to reveal definite red cell shape abnormalities. In fact, exposure of the erythrocytes to dieldrin suspensions equivalents to 0.1 mM up to 100 mM concentrations did not cause any morphological perturbation (Fig. 4).

Discussion

The observed results proved that dieldrin indeed interacts with phospholipid bilayers increasing their fluidity, particularly those of DMPC. As shown by the experiments performed on DMPC

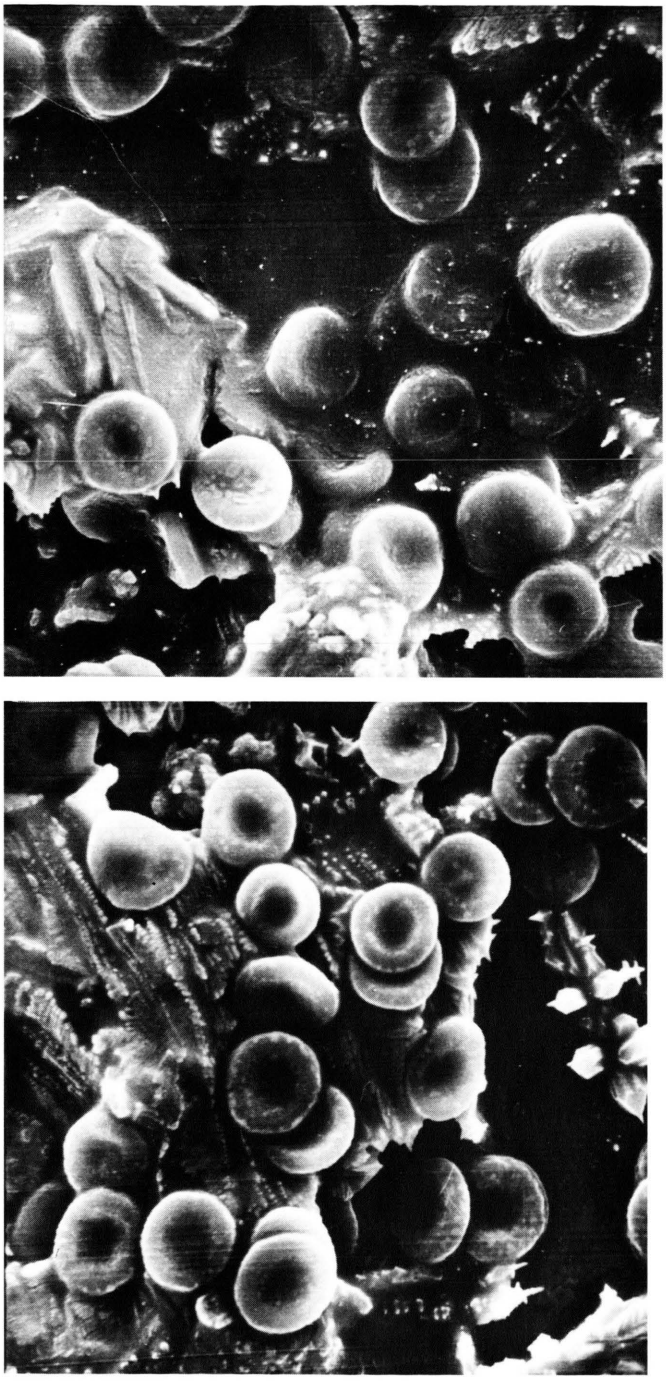

Fig. 4. Scanning electron microscope (SEM) images of human erythrocytes, 1050 x. (a) control; (b) incubated with dieldrin 100 mM.

large unilamellar vesicles (LUV), a dieldrin concentration as low as 0.01 mM induced significant perturbation to both the hydrophobic and hydrophilic regions of DMPC.

The different type and degree of perturbation induced by dieldrin to DMPC and DMPE multilayers observed in the X-ray experiments can be related to their respective packing arrangements and the effects of water upon them. Chemically they only differ in their terminal amino groups, being $+NH_3$ in DMPE and $+N(CH_3)_3$ in DMPC. Moreover, both molecular conformations are very similar in their dry crystalline phases (Suwalsky *et al.,* 1988). In fact, both have the hydrocarbon chains mostly parallel and extended with the polar groups lying perpendicularly to them. However, DMPE molecules pack tighter than those of DMPC. This effect, due to its smaller polar group and higher effective charge, results in a very stable mutilayer system which is not significantly affected by the presence of water (Suwalsky and Duk, 1987). On the other hand, the gradual hydration of DMPC results in water molecules filing the highly polar interbilayer spaces. There is, as a consequence, an increase in its bilayer width from 55 Å when dry up to about 63 Å when it is fully hydrated below its main transition temperature. This situation allowed the incorporation of dieldrin into DMPC bilayers and its penetration into the hydrocarbon chain region producing the structural disorder of the lipid. These results were confirmed by the fluorescence spectroscopy experiments performed on DMPC LUV.

It has been reported that compounds that interact with phospholipids located in the outer monolayer of the erythrocyte membrane, as it is the case of DMPC, induce a transformation from its discoid shape to a speculated one (Isomaa *et al.,* 1987). Therefore, it was expected that dieldrin would have affected the erythrocyte shape. There are, however, other pesticides such as ethylazinphos (Videira *et al.,* 1996), parathion (Antunes-Madeira *et al.,* 1994) and malathion (Antunes-Madeira and Madeira, 1993) that also interact with phospholipid bilayers and neither produced a significant structural perturbation to the erythrocyte membrane. This might be due to its high cholesterol content (about 37 mol%) (Antunes-Madeira *et al.,* 1994) which confers it a great stability. On the other hand, electrophysiological measurements performed in toad nerve-skin preparations demonstrated that dieldrin affects the function of ion channels, effect explained by its interaction with phospholipid bilayers that surround them (Quevedo *et al.,* 1997). Therefore, a molecular mechanism of dieldrin toxicity might be related to the above described results.

Acknowledgements

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